SUPPLEMENTARY DATA

EXPERIMENTAL PROCEDURES

IRP2 purification and mass spectrometry-To purify IRP2 from DFO-treated cells, Flp-In T-REx-293 cells were transiently transfected with Flag-IRP2-TEV-His. Cells were induced overnight with 1 μg/mL tetracycline in the presence of 100 μM DFO. To purify IRP2 from FAC-treated cells, stably expressing Flag-IRP2-TEV-His T-Rex-293 cells were induced overnight with 1 μg/mL tetracycline. Cells were treated with 20 μM MG132 for 30 min followed by the addition of 100 μg/mL FAC for 7.5 h. Cells were scraped into Wash Buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, containing1 mM PMSF (Sigma), cocktail phosphatase inhibitors (Calbiochem), 10 mM NaF and 25 mM β-glycerolphosphate) and lysed by sonication. Extracts were centrifuged at 18,000 x g for 15 min and then 37,000 x g for 30 min. Approximately 10 mg of cell extract was precleared using IgG beads (equilibrated with Wash Buffer) at 4°C for 1 h and was batch-purified with 100 μl prewashed M2-FLAG Agarose (Sigma) at 4°C for 2 h. Protein bound M2-FLAG Agarose was washed with Wash Buffer and then Low Salt Buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM PMSF, cocktail phosphatase inhibitors, 10 mM NaF and 25 mM β-glycerolphosphate). Flag-IRP2-His was batch-eluted in 5 column volumes Nickel Binding Buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 20 mM Imidizole, 1 mM PMSF, cocktail phosphatase inhibitors, 10 mM NaF and 25 mM β-glycerolphosphate) supplemented with 200 ng/μl 3 x FLAG peptide (Sigma) for 30 min at room temperature. Eluted protein was bound for 1 h at room temperature to Ni-NTA Sepharose 6 Fast Flow resin (GE Healthcare) that was pre-equilibrated with Nickel Binding Buffer. Ni-NTA resin was washed with Nickel Binding Buffer, then Minimal Buffer (20 mM Tris, pH 7.4, 100 mM NaCl) and Flag-IRP2-His was eluted in Elution Buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 500 μM Imidizole).

Flag-IRP2-His protein samples were further purified using a C18 Ziptip™ (Millipore), with elution into three 1-ul aliquots of 60% acetonitrile and 2% formic acid and one aliquot of 98% acetonitrile and 2% formic acid. Proteins were then digested with TPCK-modified trypsin (Promega) or chymotrypsin (Princeton) at approximately a 1 to 25 ratio (enzyme to protein) for 4 to 12 h at 37°C. Phosphopeptides were enriched by immobilized metal ion affinity chromatography (IMAC) columns charged with gallium ions (SwellGel Gallium Disc (Pierce)). The phosphopeptide enrichment method was carried out according to the Phosphopeptide Isolation Kit instructions (Pierce). LC/MS/MS analysis was performed using a LTQ-FT hybrid mass spectrometer (ThermoElectron Corp). Digest samples were introduced by nanoLC (Eksigent, Inc.) with nano-electrospray ionization (ThermoElectron Corp). Peptides were eluted from a 50-minute linear gradient from 5% acetonitrile (with 0.1% formic acid) to 60% acetonitrile (with 0.1% formic acid). Peptide molecular masses were measured by FT-ICR and peptide sequencing was performed by collision-induced dissociation (CID). Mass data peaklists for Mascot searching were generated using Sequest in Qual Browser software (Excalibur, ThermoElectron Corp.). Mascot threshold cutoffs for acceptable identified peptides (including phosphopeptides) have Mascot scores > 20, mass errors < 4 ppm, and expected values < 1.

FIGURE LEGENDS

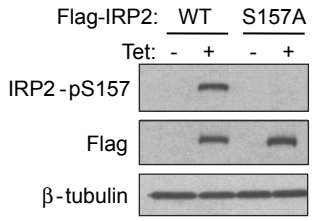
SUPPLEMENTAL FIGURE 1. **Characterization of IRP2-pS157 antibodies.** Flp-In T-REx-293 cells stably expressing WT or S157A Flag-IRP2 were induced for 16 h with tetracycline (Tet) or left uninduced. Lysates were separated by SDS-PAGE and analyzed by western blotting with anti-IRP2-pS157, anti-Flag, and anti-β-tubulin antibodies.

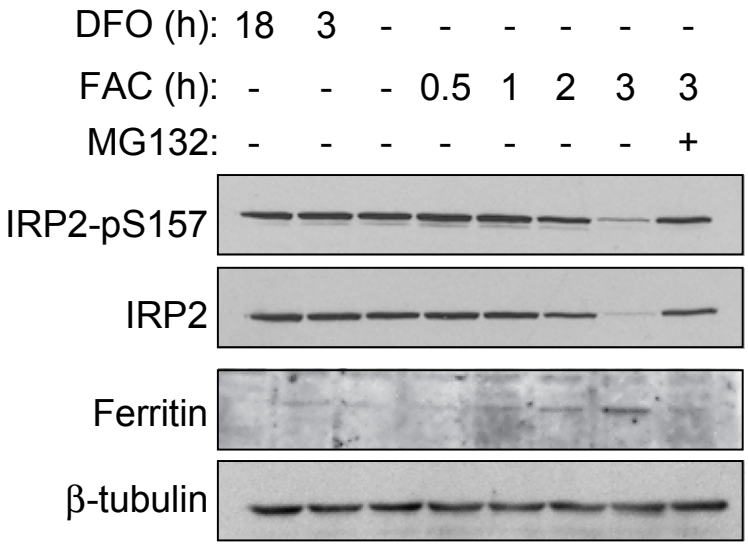
SUPPLEMENTAL FIGURE 2. **S157 phosphorylation is not regulated by iron deficiency or excess.** HEK293 cells were pretreated with 100 μM DFO (lane 1) or left untreated (lanes 2-8) for 15 h. After 15 h, one plate of cells was pretreated with 20 μM MG132 for 20 min (lane 8). Cells were then incubated for the indicated times with 100 μM DFO (lane 2), no treatment (lane 3), or 100 μg/mL FAC (lanes 4-8).

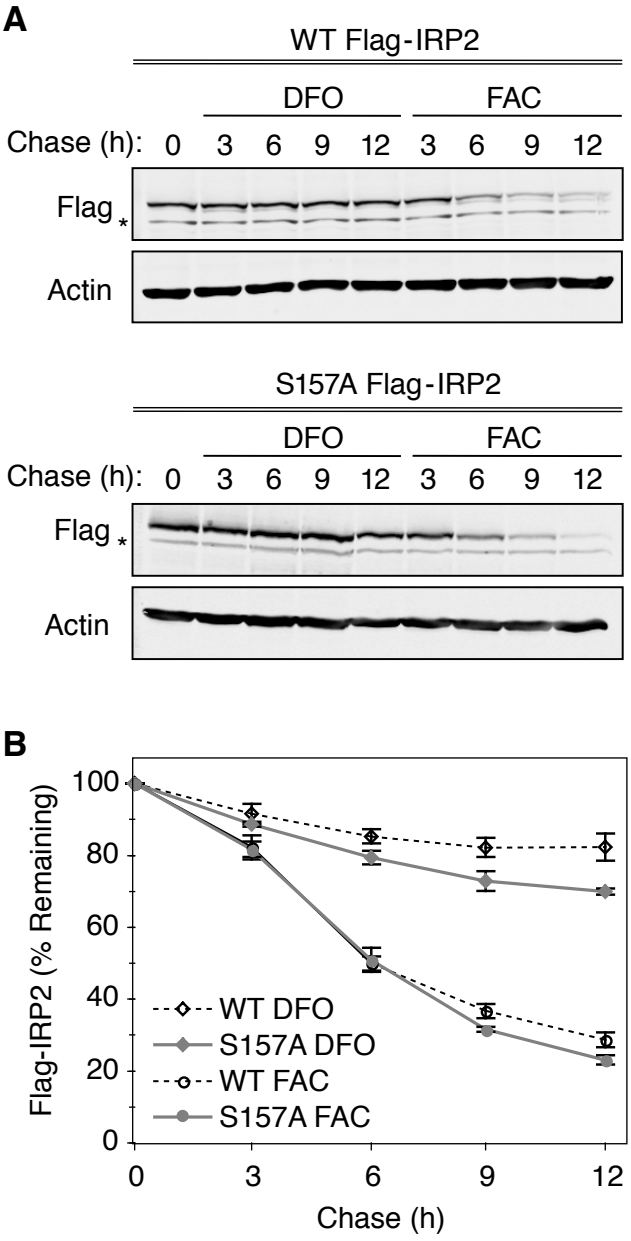
Cells were harvested simultaneously and lysates were analyzed by western blotting with anti-IRP2-pS157, anti-IRP2, anti-ferritin, and anti-β-tubulin antibodies. The results are representative of two independent experiments. These data indicate that iron deficiency or excess does not stimulate the phosphorylation or dephosphorylation of IRP2 S157. While IRP2 S157 phosphorylation decreases in extracts from cells treated with FAC for 3 h, this coincides with decreased IRP2 protein due to iron-mediated degradation. Inhibition of IRP2 degradation by treatment with the proteasome inhibitor MG132 prevents the decrease in S157 phosphorylation.

SUPPLEMENTAL FIGURE 3**. The iron-mediated degradation rate of S157A Flag-IRP2 is not altered.** Flp-In T-REx-293 cells stably expressing WT or S157A Flag-IRP2 were induced overnight with 1 ug/ml tetracycline. Cells were washed with PBS and chased in the presence of 100 μM DFO or 100 μg/mL FAC for the indicated times. (A) Lysates were separated by SDS-PAGE and analyzed by western blotting with anti-Flag, anti-actin, and anti-mouse IRDYE 800-labeled secondary antibodies (Rockland). An Odyssey Infrared Imaging System (LICOR Biosciences) was used for protein visualization and quantification. A crossreacting band is indicated by an asterisk. (B) For quantification of Flag-IRP2 degradation rates, cell extracts (5 μg) were dot blotted in triplicate onto nitrocellulose. Western blots were performed as indicated in (A) using anti-Flag and anti-mouse IRDYE 800-labeled secondary antibodies. The graph is presented as percentage of Flag-IRP2 remaining relative to 0 h. Mean half-lives (h) are reported ± the standard error with the number of independent experiments performed in parenthesis. WT Flag-IRP2 (DFO) 25.3 ± 1.3 (n = 7); S157A Flag-IRP2 (DFO) 18.7 ± 0.3 (n = 3); WT Flag-IRP2 (FAC) 7.5 ± 0.3 (n = 7); S157A Flag-IRP2 (FAC) 7.1 ± 0.2 (n = 3). These data indicate that phosphorylation or dephosphorylation of S157 does not stimulate IRP2 degradation.

Supplement Figure 1
M. Wallander, et al.




DFO (h): 18 3 - - - - - -
FAC (h): - - - 0.5 1 2 3 3
MG132: - - - - - - - +
IRP2-pS157
IRP2
Ferritin
β-tubulin


A
WT Flag-IRP2
DFO
FAC
Chase (h): 0 3 6 9 12 3 6 9 12
Flag *
Actin
S157A Flag-IRP2
DFO
FAC
Chase (h): 0 3 6 9 12 3 6 9 12
Flag *
Actin
B
Flag-IRP2 (% Remaining)
100
80
60
40
20
0
WT DFO
S157A DFO
WT FAC
S157A FAC
0 3 6 9 12
Chase (h)